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Effects of iloprost and n-acetylcysteine on ischemia-reperfusion injury and thiol/disulfide hemostasis in rats

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Abstract

Aim: Intestinal ischemia occurs after partial or complete obstruction of the intestinal arterial blood flow, and reperfusion injury following the restoration of blood flow. Intestinal ischemia-reperfusion [IIR] damage can cause multiple organ failure and death.

In our study, we aimed to observe the effect of ilioprost and N-acetylcysteine on ischemia-reperfusion injury and to show the effect of the Thiol disulfide mechanism in this area.

Material and Methods: Thirty Sprague Dawley rats were divided into five groups of six animals each: sham, IIR, IIR+IL, IIR+NAC and IIR+NAC+IL. Intestinal samples and blood were collected after completion of the sham or IIR protocol. Small-bowel samples were evaluated according to the Chiu score. Thiol/disulfide [DS] hemostasis was followed using a novel series of serum biomarkers. Serum concentrations of total thiol, native thiol and disulfide were also determined.

Results: The average Chiu score was lower in the IIR + NAC group than in both the IIR and the IIR + IL group, but the differences were not statistically significant. The score in the sham group was significantly lower than those of the other four groups. The level of reduced thiol and the native thiol/total thiol [NT/TT] ratios were higher in groups treated with NAC, IL or both. In the latter groups, oxidized thiol, DS/TT and DS/NT ratios were lower than in the IIR group but the differences between the three treatment groups were not statistically significant.

Discussion: The addition of IL to NAC was not more protective than NAC alone in a rat model of IIR injury. Our results suggest that markers of thiol-DS hemostasis can be used as indicators of antioxidant mechanisms in IIR injury.

Keywords

Ischaemia-Reperfusion Injury, Disulphide, Thiol, Iloprost

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Introduction

Ischaemia is defined as reversible or irreversible cell or tissue death due to the depletion of cell energy stores and the accumulation of toxic substances. This is the result of an insufficient blood supply to the tissues and organs due to decreased arterial and/or venous blood flow. Ischaemia causes tissue hypoxia and thus hypoxic tissue damage [1]. However, the restoration of blood flow [reperfusion] induces even more damage [2], including the formation of reactive oxygen species (ROS) from the renewed supply of cellular oxygen [3]. Mesenteric ischaemia that develops due to the occlusion of the superior mesenteric artery (SMA) prominently affects the small intestine, which is highly susceptible to ischemia-reperfusion (IR) injury [4,5]. Iloprost (IL) is a stable prostaglandin I2 analog with pharmacokinetic properties very similar to those of the parent compound [6,7]. Previous studies have demonstrated the protective effects of IL on IR injury in different organs [8]. N-acetylcysteine (NAC) is an intracellular glutathione (GSH) precursor that increases glutathione S-transferase activity in the liver [9]. Its activities include the protection of tissues against ROS damage, by directly removing oxidants [10,11]. The beneficial effects of NAC in rats, including rat models of IR injury of the heart and liver, have been demonstrated [11,12]. Thiols (-SH) are organic compounds that contain a sulfhydryl functional group [13]. Among the thiols found in plasma are albumin and other proteins, cysteine, cysteinyl glycine, glutathione, homocysteine and γ-glutamyl cysteine thiols [14]. Oxidation of the thiol groups of these thiol-containing molecules results in the formation of reversible disulfide (DS) bonds [15]. The reversible oxidation-reduction of the thiol groups and DS bonds is referred to as thiol-DS hemostasis [16] and it can be followed using the method developed by Erel and Neşelioğlu[17], which allows each and/or all of the components to be measured and further analyzed. In this study, we used of this method to investigate the abilities of IL and NAC, alone or in combination, to prevent intestinal IR (IIR) injury in rats.

Material and Methods

Experimental Design

Ethical approval was obtained from the Local Ethics Committee on Animal Experiments of Sakarya University University on 05/09/2018 with decision No. 24. Thirty mixed-sex Sprague Dawley rats weighing 250–350 g each were divided into five groups of six animals: sham, IIR, IIR+IL, IIR+NAC and IIR+NAC +IL. Prior to the experiment, the rats were acclimated under 12 h light/12 h dark conditions, with water and standard rat food supplied ad libitum. Intestinal samples [5 cm of ileum from the distal end of the terminal ileum to the proximal ileum] and blood were collected from the sham group, which did not undergo any surgical procedures. In the IIR group, the cranial mesenteric artery [CMA] and the superior mesenteric artery (SMA) were dissected and then clamped to induce intestinal ischemia. Then the skin was closed with skin staples. After 45 min of ischemia, the abdomen of the rats was reoperated and reperfusion was induced by declamping the CMA with the skin again closed using skin staples. After 120 min of reperfusion, the abdomen was reoperated and the animals were euthanized by cervical dislocation. Intestinal samples and blood were collected. In the IL-treated rats, 2 μg IL/kg was administered intraperitoneally 30 min before reperfusion. In the NAC-treated rats, 300 mg NAC/kg was administered intraperitoneally 30 min before reperfusion. Intestinal samples and blood were collected as in the sham group.

Histopathological evaluation

Tissues separated for histopathological examination were fixed in 10% formaldehyde and sent to the Department of Pathology. After routine processing, the tissue samples were embedded in paraffin, from which sections 5 μm thick were cut and stained with hematoxylin and eosin. Sections were evaluated by light microscopy (Nikon Eclipse-Ni Y-THPL, Japan) by a pathologist blinded to the treatment groups. The tissues were classified using the system of Chiu et al. (1970), described in Table1.

Biochemical Evaluation

Thiol-DS hemostasis measurements

The concentrations of total thiol (TT, μmol/L) and native thiol (NT, μmol/L) in serum were measured and the values were used to calculate the thiol/DS ratio, an oxidative marker of hemostasis. Samples collected in capped gel tubes and transferred in a cold chain were allowed to stand for 30 min before they were centrifuged (cooled, 4000 rpm 10 min). Serum parameters of interest were analyzed using Rel assay diagnostics kits. A fully automated autoanalyzer (Beckman Coulter, chemistry analyzer AU 680, serial number: 2016024580, MishIMA K.K, Japan) in the Biochemistry Laboratory was used to measure TT, NT and DS concentrations (μmol/L), from which DS/NT, DS/TT and NT/TT ratios, as well as oxidation reduction rates, were determined.

Statistical analyses

All data were processed using IBM Statistical Package for the Social Sciences Statistics 22 (SPSS 2013). Numerical variables were analyzed using descriptive statistics [mean, standard deviation], and differences between categorical variables from more than two groups were analyzed via one-way ANOVA. A Levene test was performed to determine the presence or absence of variance homogeneity, followed by a Tukey test or Tamhane's T2 test.

Ethical Approval

Ethics Committe approval for the study was obtained.

Results

Histopathological Results

A comparison of the Chiu scores of the five groups (Table 2) showed that they were lower in the sham group than in all other groups. Microscopic examination of the small intestine sections of the sham group showed that all were of grade 0. Thus, there were no morphological changes in the lamina propria, and ulceration, mononuclear cell infiltration, increased capillary permeability and hemorrhage were not observed (Figure 1a). In sections of small intestine with grade 1 damage (IIR+NAC and IIR+NAC+IL), subepithelial separations were present at the upper end of the villi (Figure 1b). In rats with grade 2 damage, the subepithelial separations were of moderate grade (Figure 1c). Grade 3 damage, seen in all IIR groups, consisted of deformations at the ends of the villi where the mucosal epithelium was pushed intensely upwards (Figure 1d). In rats with grade 4 damage (IIR, IIR+IL, IIR+NAC+IL), villus deformation was accompanied by dilated capillaries reaching

Table 1. The Chiu score.

GRADE	FINDING
0	Normal mucosal villi
1	Subepithelial separations at the upper end of the villi with capillary congestion
2	Moderate-intensity appearance where subepithelial separations push up the mucous epithelium
3	Subepithelial separations are largely observed, deformations of the villi ends where the mucosal epithelium is intensely pushed upward throughout the villi
4	Villus deformation reaching the lamina propria with dilated capillaries
5	Ulceration of the lamina propria, impaired integrity and haemorrhage

Table 2. Histopathological results [Chiu score].

GROUPS	GRADE 0	GRADE 1	GRADE 2	GRADE 3	GRADE 4	GRADE 5	TOTAL	MEAN	SD
SHAM	6						6	0.0000	0.00000
IIR			2	2	1	1	6	31.667	116.905
IIR+IL				3	3		6	35.000	0.54772
IIR+NAC		1	1	4			6	25.000	0.83666
IIR+NAC+IL		1	1	3	1		6	26.667	1,.3280

Table 3. Thiol-disulfide hemostasis in sham, IIR, IL, NAC and IL+NAC rats.

GROUPS	Sham		IIR		IIR+IL		IIR+NAC		IIR+NAC+IL		p
SIGNS	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Total thiol [TT]	205.000	52.35647	142.500	34.08665	194.333	67.21210	567.000	143.92776	390.0	91.34769	0.000
Native thiol [NT]	118.1667	53.34573	77.8333	31.85854	146.8333	63.13293	469.0000	164.12922	289.3333	83.08470	0.000
Disulphide [DS]	43.4167	6.25633	32.3333	8.26841	23.7500	4.46934	49.0000	12.89186	50.3333	9.04802	0.000
DS/TT	22.2449	5.80393	23.1603	5.42179	13.2255	4.36501	9.7009	5.35940	13.2451	2.35838	0.000
DS/NT	43.2570	18.39852	47.1421	24.30145	18.8623	8.95626	13.0991	9.64806	18.2421	4.19056	0.001
NT/TT	55.5102	11.60786	53.6795	10.84358	73.5489	8.73003	80.5983	10.71879	73.5097	4.71675	0.000
Reduced thiol ratio	55.5167	11.58886	53.7000	10.83753	73.5500	8.75506	80.6000	10.73182	73.5333	4.71876	0.000
Oxidized thiol ratio	22.2333	5.81435	23.1833	5.43338	13.2167	4.36917	9.7167	5.36150	13.2667	2.36107	0.000
Thiol oxidation reduction rate	280.4500	143.20245	251.0167	102.30494	624.1167	258.82285	1076.2667	565.89430	579.0167	162.89733	0.000

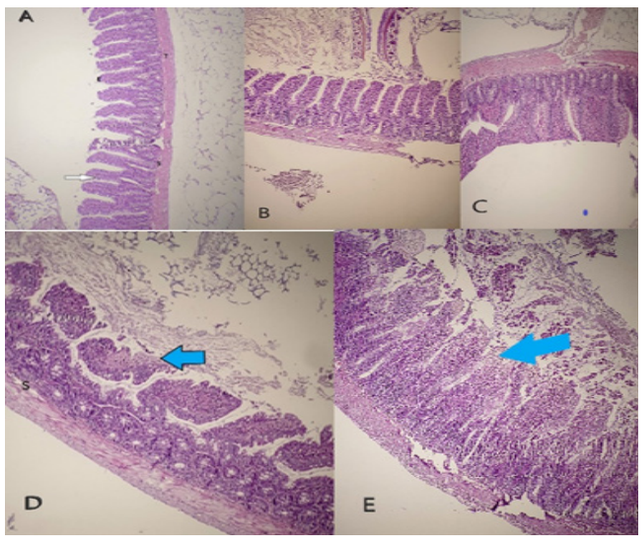

Figure 1. Rat small intestine microscopic histopathological images

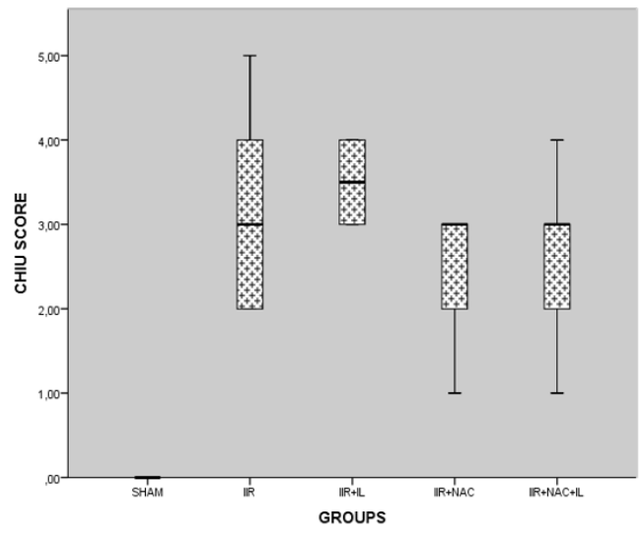

Figure 2. Histopathological evaluation of small bowel tissue with the Chiu Score

the lamina propria (Figure 1e).

IIR scores were lower in the IIR + IL group than in the IIR + NAC and IIR + NAC + IL groups, but the differences were not statistically significant (p> 0.05). All IL groups had a higher mean Chiu score than the IIR group, but again the difference was not significant. The IIR + NAC group had a lower Chiu score than either the IIR group or the IIR + IL group, but neither difference was statistically significant (Figure 2). The Chiu score of the IIR + NAC + IL group was lower than that of the IIR and IIR + IL groups and higher than that of the IIR + NAC group, without statistically significant differences. The mean Chiu score of the IIR + NAC + IL group was significantly higher than that of the sham group.

Thiol-Disulfide Hemostasis

The results of biochemical analyses of thiol-DS hemostasis in the five groups of rats are presented in Table 3.

According to the results of one-way ANOVA, TT, NT, DS, NT/TT, DS/TT, DS/NT, the reduced thiol ratio, the oxidized thiol ratio and the thiol oxidation reduction rates differed significantly among the five groups (p < 0.05). The data were further analyzed in a Levene test followed by a Tukey test for homogeneous groups and a Tamhane T2 test for non-homogeneous groups.

TT levels (Figure 3) were lower in the IIR and IIR + IL groups than in the sham group, but without a significant difference (p> 0.05). However, they were significantly higher in the IIR + NAC and IRR + NAC + IL groups than in the other groups and in the IIR + NAC group than in either the IIR + IL or the IIR + NAC + IL group.

Similar to TT, NT levels (Figure 3) were lower in the IIR and IIR + IL groups than in the sham group but the difference was not significant (p> 0.05). The levels were significantly higher in the IIR + NAC and IIR + NAC + IL groups than in the sham and IIR groups, but significantly lower in the IIR + IL group than in the IIR + NAC group.

DS levels (Figure 3) were significantly higher in the sham, IIR + NAC and IIR + NAC + IL groups than in the IIR + IL group (p <0.05), which was slightly higher than in the IIR group. The difference between the IIR + NAC + IL group and the IIR group was significant.

The reduced thiol ratio was significantly higher in the IIR + IL, IIR + NAC, and IIR + NAC + IL groups than in the sham and IIR groups (p <0.05). It was higher, but not significantly, in the IIR + NAC group than in the IIR + NAC + IL and IIR + IL groups. The NT/TT ratio was significantly higher in the IIR + IL, IIR + NAC, IIR + NAC + IL groups than in the sham and IIR groups and was also higher in the IIR + NAC group than in the IIR + NAC + IL and IIR + IL groups, but the differences were not significant.

The oxidised thiol ratio was significantly lower in the IIR + IL, IIR + NAC, IIR + NAC + IL groups than in the sham and IIR groups (p <0.05) but the difference between the IIR + NAC + IL and IIR + IL groups was not statistically significant. The DS/NT ratio was significantly lower in the IIR + NAC, IIR + IL and IIR + NAC + IL groups than in the IIR group; significantly higher in the IIR + NAC group than in the sham group; and non-significantly lower in the IIR + NAC group than in both the IIR + NAC + IL and IIR + IL group.

The DS/TT ratio was significantly lower in the IIR + IL, IIR + NAC, IIR + NAC + IL groups than in the sham and IIR groups and non-significantly lower in the IIR + NAC group than in the IIR + NAC + IL and IIR + IL groups.

The thiol oxidation reduction rate was significantly higher in the IIR + NAC + IL group than in the IIR group, and non-significantly higher in the sham and all treated groups than in the IIR group.

Discussion

Our study uniquely evaluated thiol-DS hemostasis in a rat model of IIR injury. Although methods to reduce IR injury and prevent organ failure have been described in the literature [19], our study is the first to compare the effects of IL and NAC on reperfusion subsequent to intestinal ischemia.

The protective effects of IL on ischemic musculoskeletal damage and in heart disease, lung diseases, and IR-associated spinal cord injury have been demonstrated in several studies [20-25]. Using the Chiu score, we found that IL reduced IR injury in the small intestine compared to the untreated (IR) control, but the difference was not statistically significant.

However, our study is the first to investigate the effects of these two drugs on intestinal IR. A comparison of IL and NAC, alone or in combination, showed better histopathological results in NAC-treated groups and worse histopathological results in IL groups. This finding supports previous studies that have reported that NAC may be effective for the prevention of IIR injury. Thus, the therapeutic effects of NAC in the clinical setting merit investigation.

Whether IL and/or NAC, as antioxidants, have an effect on thiol-DS hemostasis, a new marker of oxidative stress was also examined in this study. While many clinical conditions disrupt the thiol-DS balance, it is not known whether this also occurs in IIR injury. Erel and Neselioğlu [17] showed that plasma levels of DS were higher in patients with degenerative diseases such as diabetes, obesity and pneumonia as well as in smokers, but lower in patients with proliferative diseases such as multiple myeloma, bladder cancer, colon cancer and kidney cancer

Our study revealed significant differences between groups with respect to serum markers for thiol-DS haemostasis. Specifically, TT, NT and DS levels were highest in the NAC group and lower in the NAC+IL groups. The oxidised thiol level was the lowest and the reduced thiol ratio was the highest in the NAC group, which suggests that IL reduces the effects of NAC when the two drugs are administered in combination. Our results demonstrate that utility of TT, NT and oxidized and reduced thiol ratios in assessing the degree of antioxidant activity during IIR injury. This novel finding should be examined in further studies. The limitation of this study is that the number of subjects in the study was small.

Conclusion

A combination of NAC and IL has no advantage over NAC alone in protector IIR injury. The use of the Thiol disulfide mechanism and measurement parameters in intestinal ischemia-reperfusion injury gives results related to the rate of damage. New studies are needed to increase the level of evidence of the results.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest
None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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